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(54) Antigen-linked competitive enzymeimmunoassay

(57) Antigens including haptens are determined by an assay method ("ALICE", trade mark of Farmitalia Carlo Erba S.p.A.), which comprises (1) labelling (i) a known antigen (Ag^1) covalently bound to a reactant (X) for the known antigen to form a conjugate (Ag^1-X) and (ii) a sample of an antigen (Ag^2) to be determined, by reactions (a) of Ag^1-X and Ag^2 respectively, with a first antibody (Ab^1), in which the total Ag^1 and Ag^2 is in excess over Ab^1, antibody Ab^1 being specific to the antigens Ag^1 and Ag^2 and raised in an animal, and (b) between Ab^1 and a second antibody (Ab^2), which antibody Ab^2 is an antibody to immunoglobulin or a fragment thereof of the animal species in which the first antibody (Ab^1) was raised and is enzyme-labelled (2) reacting the products of (1) in the liquid phase, with immunoadsorbent comprising a solid material carrying bound on its surface a binding partner Y capable of binding X and inert to the antigen Ag^2, in a stoichiometric excess with respect to X; (3) separating the solid phase from the liquid phase; and (4) determining the enzyme activity in either or both phases. Preferably the labelling of the antigens involving reactions (a) and (b) described above is carried out before Ag^1-X is reacted with immunoadsorbent.

A test kit of enzyme-immunocomplex Ab^1-Ab^{2*} (*representing the enzyme residue bonded to Ab^2), the conjugate Ag^1-X and the immunoadsorbent is also included in the invention.

GB 2 084 317 A

SPECIFICATION

Antigen-linked competitive enzymeimmunoassay

This invention relates to an antigen-linked competitive enzymeimmunoassay and provides a novel method for the detection of antigens, including haptens. It also includes a test kit for use in this method. We refer to our method and test kit by our trade mark "ALICE".

The term "antigen" as used herein means not only substances which are capable by themselves of provoking the production of antibodies in animals, i.e. immunogens, but also substances, sometimes called haptens, which, after conjugation with a carrier molecule, become capable of provoking the production of specific antibodies.

Our British Patent No. 1,549,069 describes a method for the detection and assay of antigens, using an enzyme label. This assay involves basically comparing the reaction of a known antigen (Ag^1) which is insolubilised, for example bound to a support material, with that of the antigen (Ag^2) to be determined (detected or assayed), for the same labelling system. The labelling system consists of a first antibody (Ab^1) which is reactive with the insolubilised, known and liquid phase unknown antigens, a second antibody (Ab^{2*}) which is anti- to the first antibody and which has an enzyme label attached thereto, preferably by covalent bonding. After separating the solid and liquid phases, the enzyme activity is determined in either phase. The whole labelling system Ab^1—Ab^{2*} is conveniently termed the enzyme immunocomplex or "EIC".

The EIC incorporates a "universal label". The first antibody (Ab^1) of the EIC has to be specific to the antigens (Ag^1 and Ag^2). It can be produced by injecting the known antigen Ag^1 into an animal, for example a sheep, and exsanguinating the animal after a suitable period. The second antibody Ab^2 is produced by injecting the immunoglobulin or Fc fraction of the immunoglobulin of the first animal into a second species of animal, for example a donkey, and exsanguinating the animal after a suitable period. The illustrated second antibody is called "donkey anti-sheep IgG" for short. It is then reacted with the enzyme to label it. The labelled second antibody Ab^{2*}, illustrated as "donkey anti-sheep IgG*", can be used virtually regardless of the type of antigen. One need only inject the antigen into a sheep when producing the first antibody, since this "universal label" Ab^{2*} will react with any antibody Ab^1 raised in a sheep. There is, therfore, no need to provide a wide variety of enzyme-labelled antibodies, as was necessary in some previous assay methods and the problems inherent in linking a particular kind of enzyme to an antibody are required to be solved only once.

This prior method can be summarised schematically as:

$$\text{Support} — \left.\begin{matrix} Ag^1 \\ Ag^2 \end{matrix}\right\} — Ab^1 — Ab^{2*},$$

the parenthesis denoting a comparative procedure (sometimes called a "competitive procedure") and the asterisk the enzyme label.

In the preferred way of carrying out the above method the EIC is pre-formed. (It can frequently be made in a form which is stable for several months). The assay is then carried out merely by incubating excess EIC with the sample of antigen Ag^2 to be determined, then incubating the insolubilised, known antigen (termed the "immunoadsorbent") with the reaction product containing unreacted EIC, separating solid and liquid phases, washing the solid phase, and determining the enzyme concentration in the solid phase, which is inversely proportional to the amount of antigen Ag^2 to be determined. This method has the great advantage over previous methods of requiring only one separation step to be performed "on the site", e.g. in the hospital laboratory. Most of the known-how, including purification of reagents, is incorporated into the assay by the manufacturer who produces the immunoadsorbent and the EIC as a "test kit".

Our above prior method introduced several advantages, besides the use of an universal label and the single separation step, viz; the possibility of multiplying the signal, the lack of chemical modifications of the antigen and antibody involved in the primary reaction and other advantages generic to use of an enzyme instead of a radio-active label.

We have now found that in this system of assay the separation step can be critical. The addition of a solid phase on which large amounts of antigen Ag^1 are immobilised can interfere seriously with the reaction equilibrium between the Ab^1 of the unreacted EIC and the antigen Ag^1. This is especially true for haptens where the dissociation constant of the Ag—Ab^1 reaction is rather high. On the other side, if we decrease the amount of antigen immobilised on the solid phase the required incubation time becomes increasingly longer and the zero-binding activity is considerably lower and therefore more difficult to measure.

Another problem lies in the detection of expensive substances, for example LH (lutenizing hormone), FSH (follicle-stimulating hormone, TSH (thyroid-stimulating hormone), or HGH (human growth hormone). The preparation of immunoadsorbents bearing such substances would be extremely expensive, because of the amount of the antigen needed on the solid phase.

For every antigen to be detected in the prior method we had to prepare a specific immunoadsorbent and, on meeting the above problems each time, have had to face again and again the

need to optimise conditions so as to try to reduce the amount of antigen required without weakening the measurable enzyme activity too seriously.

A method for the assay of haptens has been described by Sadeh et al. in Journal of Immunological Methods *28*, 125 (1979) and in German Offenlegungsschrift 2,811,537. In this method a known hapten H^1 is conjugated with a substance X to form a conjugate H^1—X. The hapten conjugate H^1—X and hapten H^2 to be determined are then reacted with anti-hapten Ab^h on a solid carrier in the manner of a heterogeneous assay. The solid phase is then separated and labelled. The label is an antibody Ab^* which is anti-X and which is enzyme-labelled. The enzyme activity of the solid phase is inversely proportional to the amount of hapten H^2 to be determined, as in our prior method. This modification does not solve the problems, presenting the following disadvantages:

1) The modification uses an antibody specific to the haptens immobilised on the solid phase. This is the limiting reagent, i.e. the reagent in deficiency, the amount of which must be exactly identical in all test samples to ensure a good performance of the assay. It is difficult to prepare this reagent in a standardized way.
2) Reaction on the solid phase suffers from similar problems to those of the above method described in our prior patent. Chiefly, the speed of the reaction is low because of diffusion problems, (because the solid phase antibody is in deficiency with respect to the antigens).
3) A different anti-hapten Ab^h on the solid carrier is needed for every assay type, i.e. according to the nature of the hapten.

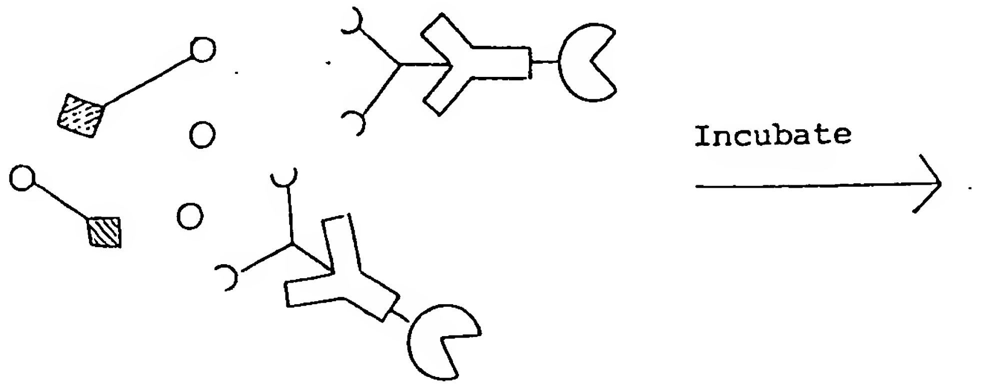
Keeping in mind all these problems, we have developed a new method which, while exploiting the advantages of the prior method, does not cause interference in the equilibrium of the primary immunological Ag—Ab^1 reaction. Like the prior methods it can be termed "competitive" (or comparative) and heterogeneous.

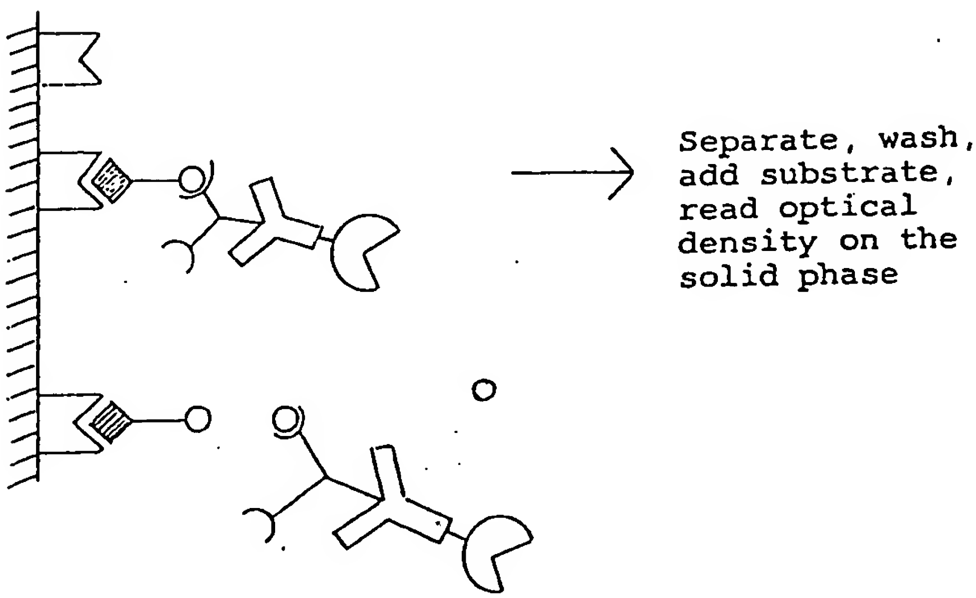
The present invention provides a method of enzyme-linked immunoassay for determining an antigen, which comprises (1) labelling (i) a known antigen (Ag^1) covalently bound to a reactant (X) for the known antigen to form a conjugate (Ag^1—X) and (ii) a sample of an antigen (Ag^2) to be determined, by reactions (a) of Ag^1—X and Ag^2 respectively, with a first antibody (Ab^1), in which the total Ag^1 and Ag^2 is in excess over Ab^1, antibody Ab^1 being specific to the antigens Ag^1 and Ag^2 and raised in an animal, (b) between Ab^1 and a second antibody (Ab^2), which antibody Ab^2 is an antibody to immunoglobulin or a fragment thereof of the animal species in which the first antibody (Ab^2) was raised and is enzyme-labelled, (2) forming a mixture of conjugate Ag^1—X and sample of antigen Ag^2 to be determined, (3) reacting the conjugate Ag^1—X in this mixture, in the liquid phase, with immunoadsorbent comprising a solid material carrying bound on its surface a binding partner Y capable of binding X and inert to the antigen Ag^2, in a stoichiometric excess with respect to X; (4) separating the solid phase from the liquid phase; and (5) determining the enzyme activity in either or both phases. Preferably the labelling of the antigens is carried out before Ag^1—X is reacted with immunoadsorbent. Thus, in the preferred method of the invention a mixture of labelled conjugate Ag^2—X and labelled sample of antigen Ag^2 is contacted with the immunoadsorbent.

The invention also includes a test kit for immunoassay to determine an antigen, comprising (a) a conjugate (Ag^1—X) of an antigen (Ag^1) covalently bound to a chemical reactant (X) for the antigen, (b) an enzyme immunocomplex which is the reaction product of a first antibody, raised in an animal, which is an antibody to the antigen, with a second antibody (Ab^2) which is an antibody to immunoglobulin or a fragment thereof of the animal species in which the first antibody was raised, and is enzyme-labelled; and (c) an immunoadsorbent comprising a solid material carrying bound on its surface a binding partner Y which is reactable with X and is inert to the antigen and/or to another antigen which is "comparable", i.e. undergoes a comparable reaction with the first antibody. (Since usually the antigen to be determined will be the same substance as the antigen Ag^1 it will be sufficient that Y is inert to Ag^1. However, in principle the antigen to be determined (Ag^2) could be slightly different from Ag^1 and the above definition of the kit covers this possibility by the alternative definition of the antigen to which Y must be inert.) Preferably the kit also includes for calibration purposes a solution containing a known amount of the antigen (Ag^2) which the kit is intended for determining.

In one method of assay the label is allowed to react with all the Ag^2 before reaction with the Ag^1—X. In another method the Ag^2 and Ag^1—X are reacted simultaneously with the label. Thus a mixture of Ag^1—X and Ag^2 can be incubated with the label. In both methods all the label is reacted and the proportion of label which is reacted with Ag^1—X is ultimately taken up into the solid phase through reaction with Y.

The method is illustrated by the following diagram:—



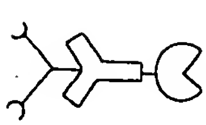
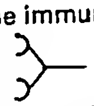
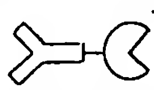


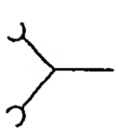
 solid material

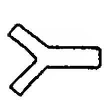
 Y, a binding partner for X

 X, a chemical reactant covalently bound to the antigen Ag^1

 antigen to be determined (Ag^2)

 pre-formed enzyme immunocomplex (EIC) constituted by a purified antibody Ab^1  immunologically bound with the "universal label"  Ab^{2*}

 antibody Ab^1 residue

 antibody Ab^2 residue

 enzyme residue

A preferred general method of carrying out the assay is as follows. An amount of Ag^1—X (chosen following the same criteria of radio-labelled antigen in radio-immunoassay) is mixed with the sample containing Ag^2 and with a limited amount of EIC (not enough to react with the total Ag^1 plus Ag^2). When the equilibrium is reached (or after allowing a suitable reaction time), a solid phase containing a large excess of Y (binder for X) is added to the reaction mixture. When all Ag^1—X (both reacted and unreacted with EIC) has been bound by Y and therefore attached to the solid phase, the supernatant liquor is discarded and the amount of enzyme of the solid phase is measured. This amount will be inversely proportional to the Ag^2 concentration in the sample.

In the reaction occurring in homogeneous (liquid) phase among: Ag^1—X, Ag^2 and EIC, the reaction time is ruled by the following factors:

a) the sensitivity required for the specific assay
b) affinity of the Ab^1 antibody for the antigens
c) decision to perform the assay at the equilibrium or not (which depends on the precision required in the assay).

The heterogeneous reaction (solid-liquid) to remove the complexes EIC—Ag^1—X by the Y-solid phase involves an interaction completely independent of the reaction previously described. Therefore the amount of Y is not limited by the necessity of not interfering in the reaction equilibrium of the homogeneous phase reaction. It can vary from a bare excess to a large excess. The greater the excess of Y the faster the reaction proceeds, and there is therefore a conflict of advantage in economising in the amount of Y and obtaining fast separation. Usually the minimum amount of Y to reach reaction equilibrium within half an hour is a fair balance of these advantages. Also, there are good prospects of using Y substances with much higher affinity constants, than is usual for antibody-antigen reaction. For example, the biotin-avidin system could be used: avidin (Y) has a high affinity constant for biotin (X). The advantages of this system are mentioned hereinafter. However, the invention includes, as a specific embodiment, the case in which Y is other than avidin and X is other than biotin.

The steps defined above for the process of the invention can be carried out in any feasible order. Thus it is possible to react the mixture of conjugate Ag^1—X and sample of antigen Ag^2 before or after labelling the antigens with the Ab^1—Ab^{2*} system, or even simultaneously if reaction conditions appropriate to both reactions are chosen.

The labelling is preferably carried out using the pre-formed enzyme-immunocomplex (EIC), Ab^1—Ab^{2*}, but it is possible to react the antigens sequentially with the specific antibody Ab^1 and then add the "universal label" Ab^{2*}.

The enzyme activity is preferably read on the solid phase, in which case it will be inversely proportional to the amount of antigen to be determined.

The EIC is in general terms the same reagent as in our prior patent mentioned above, and can be any of the specific EICs described in our prior patent mentioned above. As explained in our prior patent, the antibodies of the EIC are preferably purified from interfering reactants, e.g. by affinity chromatography.

Antigen Ag^1 can be any which is "comparable", i.e. either the same as the antigen Ag^2 or any other antigen which is sufficiently similar to Ag^2 to react with the antibody Ab^1. X must be chosen to provide a stable linkage between it and the antigen. Ordinarily this will be a covalent one. The conjugate Ag^1—X must not interfere with recognition of the antigen Ag^1 by the anti-Ag specific antibody Ab^1. X must be substantially absent (trace amounts are usually tolerable) from the biological fluid on which the assay is to be carried out (plasma, serum, urine, for example) and must be non-interfering (or only weakly interfering) with the sample components. All these requirements are inherently evident from the above definition of the invention. Further, X is preferably:

1) a well defined and stable molecule
2) easily chemically modifiable in order to obtain the maximum reaction yield with the antigen Ag^1 smoothly and therefore save on expense antigens, and
3) preferably immunogenic (or transformable into an Immunogenic substance) so that an anti-X antibody can be used as the X-binder "Y".

Y preferably has a very high affinity for X. Y will ordinarily be a protein, in order to obtain easy insolubilisation. Optimally Y will be cheap, easily available, preparable and standardizable.

In one preferred aspect of the invention, X is an animal protein absent from human body fluids, for example rabbit IgG, covalently bound to the antigen Ag^1 and Y is an antibody raised against the animal protein in a different animal species, for example donkey anti-rabbit IgG.

In another preferred aspect, applicable particularly when the antigen is a small molecule such as triiodothyronine (T_3), X is DNP, bound to the antigen Ag^1, if necessary through a coupler, and Y is an antibody to DNP. Y can be raised from a DNP (dinitrophenolated) — protein conjugate. The coupler can conveniently be gamma-aminobutyric acid when the antigen is T_3.

In another embodiment, X is biotin and Y is avidin. The system biotin-avidin as X—Y would have the following advantages:

1) Avidin has very high affinity for biotin: Coefficient of affinity = 10^{15} (cf. Ag—Ab 10^{5}—10^{11}; Protein A—Fc fragment of IgG; Lectin-Sugars 10^{3}—10^{4})
2) The affinity does not change when biotin is chemically modified in its carboxylic acid residue or when it is immobilised on a solid support
3) Avidin can be easily adsorbed on glass or polystyrene
4) Biotin is very easily chemically modifiable
5) Avidin is readily available or purifiable, and is quite stable below pH 9.0;
6) Avidin is not an immunoglobulin and therefore cross reactivity with the labelled anti-IgG present in the EIC cannot occur;
7) Being available in large homogeneous batches, avidin is easier to standardise than antibodies. Biotin is also easy to standardise.

All the main advantages of our prior method described above are still valid in the method of the invention. Furthermore the following additional advantages must be considered:

1) The insolubilised binder Y is a "universal reagent", being independent of the antigen to be measured.
2) The amount of Y is in excess of the amount of X and therefore of antigen Ag^1 and therefore its preparation is less critical in relation to the problems of standardisation and interbatch variations encountered in the method of our prior patent.
3) The separation step is quite fast.
4) The faster separation gives rise to the possibility to set up automated or semi-automated systems.
5) Ag^1—X, especially when X is an hapten, being of low molecular weight and chemically well characterised molecule, will be easily reproducible in different preparations.
6) The amount of Ag^1—X, used in the assay, is very low: this involves the possibility of applying the assay to determining expensive antigens.
7) The preparation of Ag^1—X using different arms or linkage points of the molecular structure gives the possibility of calibrating the affinity of the conjugate towards the Ab^1 antibody, optimising it to the sensitivity and specificity required by the assay.

The differences between the method of the invention and our prior method can be summarised by the following short-hand notation, using the same symbols as above:

Method of the invention

$$\left.\begin{array}{ll} \text{Support—Y—X—} & \text{Ag}^1\text{—} \\ \text{(excess)} & \text{Ag}^2\text{—} \end{array}\right\} \text{Ab}^1\text{—Ab}^{2*}$$

Method of our above-mentioned prior patent

$$\left.\begin{array}{ll} \text{Support—} & \text{Ag}^1\text{—} \\ & \text{Ag}^2\text{—} \end{array}\right\} \text{Ab}^1\text{—Ab}^{2*}$$

The differences between the method of the invention and the Sadeh *et al*. method described above in relation to assay of haptens can be summarised conveniently by the following short-hand notation, using the same symbols as above:

Method of invention

$$\left.\begin{array}{ll} \text{Support—Y—X—} & \text{H}^1\text{—} \\ \text{(excess)} & \text{H}^2\text{—} \end{array}\right\} \text{Ab}^1\text{—Ab}^{2*}$$

Sadeh et al method

$$\text{Support—Ab}^h \left\{\begin{array}{l} \text{—H}^1\text{—X—Ab}^* \\ \text{—H}^2 \end{array}\right.$$

Ab^h represents the specific anti-hapten while Y represents a substance which need only be specific to the hapten partner X, not to the hapten itself.

The following Examples illustrate the invention. All concentrations and percentages are by weight. "nm" means nano moles, i.e. 10^{-9} molar.

Triiodothyronine (T_3) was chosen as model hapten because its assay has very stringent requirements and limitations due to the very low T_3 serum levels and to the comparatively much higher serum levels of tyroxine (T_4), a very similar and potentially cross-reacting substance. We reasoned that if the assay could reach the sensitivity needed for T_3, it could be expected to be applicable with greater ease to many other haptents.

To test the effect of the nature of the reactant "X" on the assay, we chose two very different substances as "X": a large protein: rabbit-IgG, and a small hapten: a dinitrophenol (DNP).

EXAMPLE 1

Components

Ag^2	is T_3
Ag^1—X	is a T_3-Rabbit IgG prepared by the carbodiimide method and having a molecular ratio T_3/Rabbit IgG = 2/1
Ab^1	is an anti-T_3 antibody, raised in sheep and purified by immunoaffinity chromatography
Ab^{2*}	is an anti-sheep IgG antibody raised in a donkey and labelled with the enzyme β-galactosidase from *E. Coli*
EIC	is constituted by Ab^1 + Ab^{2*} mixed together until the reaction is complete and stored at 4°C until used in the assay
Support—Y	is an anti-rabbit-IgG antibody raised in donkey and covalently coupled to microcrystalline cellulose
Buffer	is a 50 mM Barbitone Buffer, pH 8.6, containing 0.2% gelatin and 0.3 M NaCl

Analytical method

EIC (—Ab^1—Ab^{2*}) (200 μl), T_3 standard solution in buffer (Ag^2) (100 μl) and T_3-Rabbit IgG conjugate (Ag^1—X) (100 μl of a dilution containing 100 nM/litre of the conjugate) were mixed and incubated for 2 hrs at room temperature. A suspension of cellulose-anti rabbit-IgG (Support—Y) (200 μl containing 1 mg/ml of solid phase) was added and the mixture incubated for 1 hour at room temperature.

The solid-phase was separated and washed by centrifugation. The amount of β-galactosidase bound to the solid-phase was measured by a colorimetric assay using *o*-nitrophenyl-β-galactopyranoside as substrate (Craven et al., J. Biol. Chem. *240* 2468 (1965)

Results

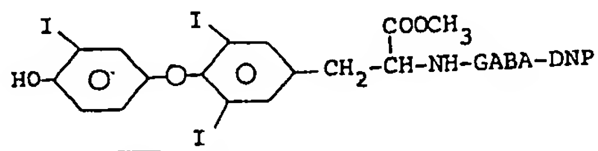
T_3 concentration (nM/litre)	Change (Δ) in Optical Density (after 30 minutes reaction) (Duplicate readings)	Average % of zero binding
0	0.519 ; 0.545	100
0.5	0.534 ; 0.532	100
1	0.508 ; 0.499	93
2	0.461 ; 0.405	78
4	0.366 ; 0.366	63
8	0.267 ; 0.282	42
16	0.222 ; 0.214	30
32	0.187 ; 0.185	23
NSB	0.083 ; 0.084	

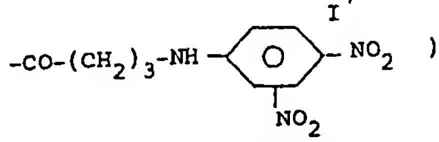
Note: NSB = Non Specific Binding, i.e. the amount of label bound to the solid phase other than through T_3-Rabbit IgG. Percentages of binding are calculated after subtracting NSB

EXAMPLE 2

Components

Ag^2, Ab^1, Ab^{2*} and EIC are the same as in Example 1. Ag^1—X is a T_3-DNP conjugate of the following formula:



(GABA-DNP = 

Support—Y is an anti-DNP antibody raised in a rabbit and covalently coupled to microcrystalline cellulose

Analytical method

The assay procedure is identical to that of Example 1 except for the Ag^1—X concentration, which was reduced to 5 nM/litre, giving a higher sensitivity.

Results

T_3 concentration (nM/litre)	Change (Δ) in Optical Density (after 60 minutes reaction) (duplicate readings)	Average % of zero-binding
0	0.452 ; 0.499	100
0.5	0.422 ; 0.458	90
1	0.320 ; 0.397	77
2	0.295 ; 0.305	50
4	0.217 ; 0.216	27
8	0.152 ; 0.130	5
16	0.137 ; 0.141	5
NSB	0.122 ; 0.122	

EXAMPLE 3

The assay of Example 2 was made even more sensitive by reducing to half the amount of EIC whilst keeping all the other reagents and procedure identical

Results

T_3 concentration (nM/litre)	Change (Δ) in Optical Density (after 60 minutes reaction) (duplicate readings)	Average % of zero-binding
0	0.289 ; 0.298	100
0.5	0.211 ; 0.224	68
1	0.167 ; 0.188	50
2	0.131 ; 0.138	32
4	0.101 ; 0.100	17
8	0.082 ; 0.088	11
16	0.069 ; 0.072	4
NSB	0.060 ; 0.060	

CLAIMS

1. A method of enzyme-linked immunoassay for determining an antigen, which comprises (1) labelling (i) a known antigen (Ag^1) covalently bound to a reactant (X) for the known antigen to form a conjugate (Ag^1—X) and (ii) a sample of an antigen (Ag^2) to be determined, by reactions (a) of Ag^1—X and Ag^2 respectively, with a first antibody (Ab^1), in which the total Ag^1 and Ag^2 is in excess over Ab^1, antibody Ab^1 being specific to the antigens Ag^1 and Ag^2 and raised in an animal, (b) between Ab^1 and a second antibody (Ab^2), which antibody Ab^2 is an antibody to immunoglobulin or a fragment thereof of the animal species in which the first antibody (Ab^2) was raised and is enzyme-labelled, (2) forming a mixture of conjugate Ag^1—X and sample of antigen Ag^2 to be determined, (3) reacting the conjugate Ag^1—X in this mixture, in the liquid phase, with immunoadsorbent comprising a solid material carrying bound on its surface a binding partner Y capable of binding X and inert to the antigen Ag^2, in a stoichiometric excess with respect to X; (4) separating the solid phase from the liquid phase; and (5) determining the enzyme activity in either or both phases.

2. A method according to claim 1 wherein X is a dinitrophenol residue covalently bound through a coupler to the antigen Ag^1, and Y is an antibody to dinitrophenol.

3. A method according to claim 1, wherein the antigen is triiodothyronine.

4. A method according to claim 1, wherein X is biotin and Y is avidin.

5. A method according to claim 1, wherein the antigen is a hapten.

6. A method according to any preceding claim wherein the antigens are labelled by reacting them with a preformed immunocomplex of the first antibody and labelled second antibody (Ab^1—Ab^{2*}).

7. A method according to any preceding claim wherein the enzyme activity is determined in the solid phase.

8. A method according to any preceding claim wherein the antigens Ag^1 and Ag^2 are labelled by reactions (a) and (b) as defined in claim 1 before the conjugate Ag^1—X is reacted with the immunoadsorbent.

9. A method according to any preceding claim wherein the antigens Ag^1 and Ag^2 are the same.

10. A method according to claim 1 substantially as described in any one of the Examples.

11. A test kit for immunoassay comprising
(a) a conjugate (Ag^1—X) of an antigen (Ag^1) covalently bound to a chemical reactant (X) for the antigen,
(b) an enzyme immunocomplex which is the reaction product of a first antibody (Ab^1), raised in an animal, which is an antibody to the antigen, with a second antibody (Ab^2) which is an antibody to immunoglobulin or a fragment thereof of the animal species in which the first antibody was raised, and of the second antibody (Ab^2) with an enzyme label; and
(c) an immunoadsorbent comprising a solid material carrying bound on its surface a binding partner Y which is reactable with X and is inert to the antigen (Ag^1) and/or a comparable antigen which is different from Ag^1.

12. A kit according to claim 11 having any one or more of the features defined in claims 2 to 6.

13. A method of enzyme-linked immunoassay incorporating the system

$$\text{Support—Y—X—}\left.\begin{matrix}\text{Ag}^1\text{—}\\ \text{Ag}^2\text{—}\end{matrix}\right\}\text{Ab}^1\text{—Ab}^{2*}$$

and test kit for use in this method, substantially as hereinbefore described.

New claims or amendments to claims filed on 5 November 1981

New or amended claims:—

1. A method of enzyme-linked immunoassay for determining an antigen, which comprises (1) labelling (i) a known antigen (Ag^1) covalently bound to a reactant (X) for the known antigen to form a conjugate (Ag^1—X) and (ii) a sample of an antigen (Ag^2) to be determined, by reactions (a) of Ag^1—X and Ag^2 respectively, with a first antibody (Ab^1), in which the total Ag^1 and Ag^2 is in excess over Ab^1, antibody Ab^1 being specific to the antigens Ag^1 and Ag^2 and raised in an animal, and (b) between Ab^1 and a second antibody (Ab^2), which antibody Ab^2 is an antibody to immunoglobulin or a fragment thereof of the animal species in which the first antibody (Ab^1) was raised and is enzyme-labelled, reactions (a) and (b) being carried out in either order or simultaneously; (2) forming a mixture of conjugate Ag^1—X and sample of antigen Ag^2 to be determined; (3) reacting the conjugate Ag^1—X in this mixture, in the liquid phase, with immunoadsorbent comprising a solid material carrying bound on its surface a binding partner Y capable of binding X and inert to the antigen Ag^2, in a stoichiometric excess with respect to X steps (1)(a), (1)(b) (2) and (3) being carried out in any order or simultaneously as feasible provided that step (2) preceeds step (3); (4) separating the solid phase from the liquid phase; and (5) determining the enzyme activity in either or both phases.

11. A method according to any one of the preceeding claims, wherein steps (1)(a), (1)(b) and (2) are carried out simultaneously followed by step (3).

12. A test kit for immunoassay comprising

(a) a conjugate (Ag^1—X) of an antigen (Ag^1) covalently bound to a chemical reactant (X) for the antigen, (b) an enzyme immunocomplex which is the reaction product of a first antibody (Ab^1), raised in an animal, which is an antibody to the antigen, with a second antibody (Ab^2) which is an antibody to immunoglobulin or a fragment thereof of the animal species in which the first antibody was raised, and of the second antibody (Ab^2) with an enzyme label; and
(c) an immunoadsorbent comprising a solid material carrying bound on its surface a binding partner Y which is reactable with X and is inert to the antigen (Ag^1) and/or a comparable antigen which is different from Ag^1.

13. A kit according to claim 12 having any one or more of the features defined in claims 2 to 5.

14. A method of enzyme-linked immunoassay incorporating the system

$$\text{Support—Y—X—}\left.\begin{matrix}\text{Ag}^1\text{—}\\ \text{Ag}^2\text{—}\end{matrix}\right\}\text{Ab}^1\text{—Ab}^{2*}$$

and test kit for use in this method, substantially as hereinbefore described.

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